Improving the Efficiency of Homologous Gene Replacement by Disrupting the Non-Homologous End Joining Pathway Gene *KusA* in *Mortierella alpina*

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Introduction

Mortierella alpina, an oleaginous filamentous fungus, is one of several industrial strains known for the production of arachidonic acid. It is also of particular interest for hydrocarbon biofuel production since it is able to produce up to 50% of its mass in rich, long-chain polyunsaturated fatty acids [PUFA's]. *M. alpina* already has mechanisms for accumulating significant concentrations of hydrocarbon compounds, making it a naturally equipped candidate to handle potential toxic concentrations of hydrocarbons.

Background

- Gene deletion occurs when knockout vectors are integrated into host genome exactly at a gene locus via homologous recombination.
- Recent studies in *M. alpina* show that gene deletion has an inherently low rate of homologous recombination.
- Prior research has shown that *kusA* deletion mutants exhibit increased rates of homologous recombination (HR) in different fungal strains such as *Neurospora* strains [1].

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Goals

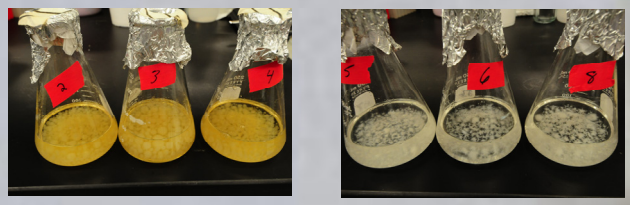
The main goal of this study was to develop a *KusA*-knock-out mutant of *M. alpina* in order to increase its homologous recombination [HR] efficiency, which will enable researchers to engineer this fungal strain for further improvement of lipid production.

The hygromycin-resistance gene was selected as the selection marker for *kusA* gene replacement.

- Optimization of cultural growth conditions by testing various media compositions and inoculation volumes;
- Optimization conditions for protoplast formation

Methods

Step 1: Culture Media Optimization



Fungal Cultures are grown in mediums of glucose, salts, nitrogen and vitamins

Step 2: Protoplast Production and Harvest

Protoplasts are individual cells with only a protective coat-plasma membrane. The goal of efficient transfer of genetic material is the basis for the formation of stable, abundant, and viable protoplasts.

The effects of some factors on protoplast isolation and regeneration:

- enzyme time
- temperature
- osmotic pressure stabilizer
- mycelia incubation time and age

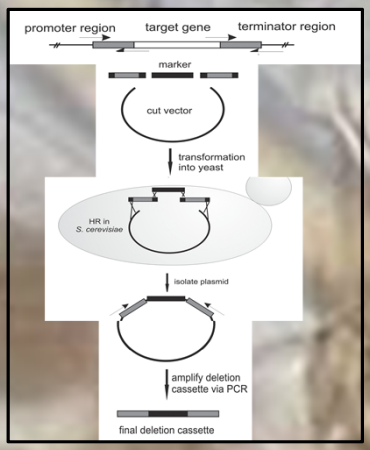
Buffer solutions tested were Mannitol, Sorbitol, and a Magnesium/Maleic Acid.

Incubation times at 30° C.

Mycelia incubated on shaker at low speed for 2 to 4 hrs for protoplast formation.

Step 3: The vector was developed by yeast-gap repair method. (See below for details on yeast gap method)

Yeast Gap Cloning Overview [4]

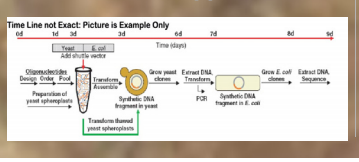


See Verification Step under Top Left Tab

Step 4: After protoplasts were produced by Step 2, they were mixed with the gene deletion Vector DNA from Step 3, and embedded in the protoplast transformation agar containing a proper amount of hygromycin.

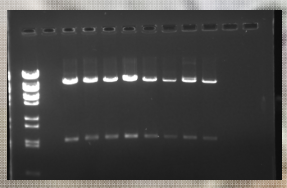
Step 5: Ongoing – Transformants regenerated from protoplasts will examined for the gene deletion.

Yeast Gap Repair-Vector Construction-Method [3]



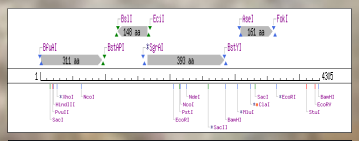
Vector Verification

PCR amplification: after vector assembly in yeast and duplication in E. Coli, presence of DNA construct was verified by PCR and specific enzymes : for example, plasmid DNA digested with BamHI below.



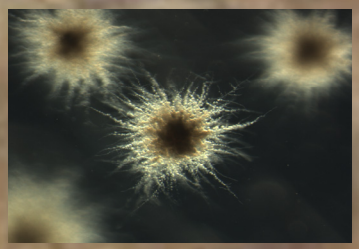
Expected Results

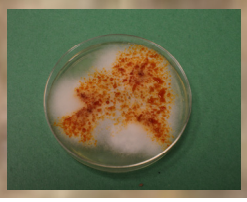
- Hygromycin transformants were obtained using the *KusA* constructs.
- Culture media for best growth and maintenance for this quick growing fungus was optimized.
- Conditions for Protoplast formation, harvest, and transformation were optimized.
- Knockout Verification-PCR.



Conclusion

- The successful deletion of *KusA* within the biotechnological important *M. alpina* will enable homologous recombination of other genes of interest in a higher frequency. This capacity may also improve the advanced production of microbial oils for bioenergy and arachidonic acid for human health applications.





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References

[1] Ninomiya Y, Suzuki K, Ishii C, Inoue H. (2004). Highly efficient gene replacements in Neurospora strains deficient for nonhomologous end-joining. PNAS 101: 12248-12253.

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[4] Colot, Hildegarde, Monika Aurélio, (2010). Molecular Genetic Manipulation of Fusarium and Prospects. Figure 4. Yeast Integrative Cloning. Methods in Molecular Biology 639

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